Influence of Bispecific Antibodies on the In Vitro Bactericidal Activity of Bovine Neutrophils Against *Staphylococcus aureus*

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ABSTRACT

We conducted the following study to determine if bispecific antibodies enhance the bactericidal activity of bovine neutrophils. Bispecific antibodies were synthesized by chemically crosslinking bovine neutrophil monoclonal antibodies to *Staphylococcus aureus* 305 capsule polysaccharide monoclonal antibodies. The efficiency of chemically coupling monoclonal antibody monomers was approximately 50% for each bispecific antibody produced. Monoclonal antibodies against neutrophils enhanced the respiratory burst activity of neutrophils by 2.3- to 2.5-fold. To determine the influence of bispecific antibodies on neutrophil function, *S. aureus* 305 was preincubated with various concentrations of bispecific antibodies and neutrophils were then added to the opsonized bacteria at different bacteria to neutrophil ratios. The bactericidal activity of neutrophils was expressed as a percentage reduction in colony-forming units in test cultures compared with the number of colony-forming units in control test cultures that did not contain bispecific antibodies or neutrophils. The addition of bispecific antibodies to test cultures increased the bactericidal activity of neutrophils. A reduction in colony-forming units as a function of increasing the *S. aureus* 305 to neutrophils ratio was observed in both the absence and presence of bispecific antibodies. However, a greater reduction was observed in the presence of bispecific antibodies. Increasing concentrations of bispecific antibodies enhanced the bactericidal activity of neutrophils at a constant *S. aureus* 305 to neutrophil ratio of 1:500. The results indicate that bispecific antibodies that recognize both *S. aureus* 305 capsular polysaccharide and neutrophil antigens potentiate the bactericidal activity of neutrophils.

(**Key words:** bispecific antibody, mastitis, *Staphylococcus aureus*, neutrophils)

Abbreviation key: BsAb = bispecific antibody, **Cp5** = *Staphylococcus aureus* 305 capsular polysaccharide type 5, **mAb** = monoclonal antibody, **PMN** = polymorphonuclear neutrophilic leukocyte.

INTRODUCTION

Mastitis is caused by bacterial infection of the mammary gland, and polymorphonuclear neutrophilic leukocytes (**PMN**) are the first immunological line of defense against intramammary infections (5). Neutrophils are phagocytic cells that engulf and kill pathogens that infect the mammary gland. Bacterial infection of the mammary gland triggers the release of inflammatory mediators that signal and facilitate the diapedesis of PMN from blood into the mammary gland. The ability of PMN to migrate from blood into the gland to phagocytose and kill bacteria directly affects the severity and duration of infection (3, 5, 9, 10).

Functional activities of PMN, such as chemotaxis, phagocytosis, and oxidative metabolism, are regulated by the appropriate stimulation of cell surface receptors. Crosslinking of L-selectin adhesion receptors on PMN with monoclonal antibodies (**mAb**) potentiates respiratory burst activity (8, 14), which is a major bactericidal mechanism (13). Consequently, mAb may provide a novel means to enhance PMN function and therefore, treat and control bovine mastitis. The incidence of mastitis and decreased respiratory burst activity of PMN are directly correlated (13).

The formation of bispecific antibodies (**BsAb**) by covalently coupling PMN mAb to mAb against bacterial pathogens that infect the mammary gland may ameliorate the bactericidal activity of PMN by decreasing the physical proximity of bacteria to PMN and increasing the respiratory burst activity of PMN. Therefore, BsAb therapy has the potential to lower the susceptibility of cows to mastitis and reduce the severity and duration of intramammary infections.

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In the present study, BsAb were synthesized by chemically coupling bovine PMN mAb to mAb that recognizes *Staphylococcus aureus* 305 capsular polysaccharide type 5 (**Cp5**, Newbould strain 305, American Type Culture Collection #29740). The objective of this investigation was to evaluate the influence of BsAb on the in vitro bactericidal activity of PMN against *S. aureus* 305.

MATERIALS AND METHODS

Synthesis of BsAb

Three bovine PMN mAb, 11G10 (IgG_1), 36H10 (IgG_{2a}), and 6C6 (IgG_{2a}) were produced as described by Salgar et al. (8). Briefly, spleen cells from mice that were inoculated with bovine PMN were fused with myeloma cells. Hybridoma clones producing mAb to PMN were injected intraperitoneally into pristane-primed mice to induce ascites. Monoclonal antibodies from ascitic fluid were purified by protein G affinity chromatography (Hi-Trap Protein G column, Amersham Pharmacia Biotech, Piscataway, NJ). The Cp5 mAb (IgG_1) was provided by B. Poutrel (6).

Three BsAb, 11G10 × Cp5, 36H10 × Cp5, and 6C6 × Cp5 were prepared by chemically coupling bovine PMN mAb and Cp5 mAb with the crosslinking compound N-succinimidyl-3-(2-pyridithiol)-propionate (11). Heteroaggregates of BsAb were separated from monomeric mAb by size-exclusion gel filtration (fast protein liquid chromatography, Superose 12 HR column, Amersham Pharmacia Biotech, Piscataway, NJ).

Isolation of Blood PMN

Blood samples from healthy, lactating Holstein cows (n = 4) from the USDA-ARS dairy herd (Beltsville, MD) were drawn into tubes containing acid-citrate dextrose, and PMN were isolated by differential centrifugation and hypotonic lysis of red blood cells (14). The concentration, differential count (92 to 96% PMN), and viability (98 to 99%) of PMN were determined by an electronic cell counter (Coulter Electronics Inc., Hialeah, FL), Wright-stained smears, and trypan blue dye exclusion, respectively. Depending on the assay performed, isolated PMN were suspended in PBS (pH 7.2) at a concentration of 1×10^6 or 1.5×10^7 cells/ml and stored at 4°C.

Respiratory Burst Activity

Respiratory burst activity of PMN was determined by luminol-amplified, native chemiluminescence (Sigma Chemical Co., St. Louis, MO) activity (14). Approximately 10^7 PMN from two cows were incubated with the bovine PMN mAb 36H10 or 6C6 or *Streptococcus agalactiae* mAb in ascites for 2 h at 4°C. The *Strep. agalactiae* mAb was used to determine nonspecific binding of Fc receptors on PMN and subsequent stimulation of respiratory burst pathway. The cell suspension and stock solution of luminol (10 m*M*) were prewarmed to 39°C for 15 min before chemiluminescence activity was measured. Chemiluminescence activity was initiated by the addition of luminol to a final concentration of 40μM and counts per minute were recorded at 15-min intervals for 120 min by a liquid scintillation counter. Each assay was performed in duplicate. The *Strep. agalactiae* mAb was produced as described above. The mAb 11G10 has been previously characterized and shown to recognize L-selectin molecules on the bovine PMN membrane (14).

Bactericidal Assay

A trypticase soy agar plate supplemented with 5% bovine blood was inoculated with a stock culture of *S. aureus* 305 and incubated overnight at 37°C. A single colony-forming unit from the plate was suspended in 1 ml of PBS and 100 μl was spread on Columbia agar supplement with 450 m*M* $MgCl_2$ and 58 m*M* $CaCl_2$. After an overnight incubation at 37°C, bacteria were harvested and washed three times with PBS. The pellet was resuspended in PBS, adjusted to 0.1 absorbance units at 520 nm (approximately 10^9 cfu/ml), and serially diluted with PBS to the appropriate concentration.

The influence of BsAb on the bactericidal activity of PMN was determined by 1) varying the bacteria to PMN ratio (bacteria only, 1:10, 1:100, or 1:500) and maintaining a constant BsAb concentration (5 μg) or 2) maintaining a constant bacteria to PMN ratio of 1:500 and varying the concentration of BsAb (0, 5, 10, 20, or 50 μg). Aliquots (100 μl) of *S. aureus* 305 (3×10^4 cfu/ml) were preincubated (30 min, 24°C) with one of the three types of BsAb in a 96-well microtiter plate (100 μl BsAb/well). The appropriate concentrations of PMN (100 μl) were added to each well and incubated an additional 60 min at 37°C on an orbital shaker (120 rpm). An aliquot from each well was then serially diluted and sonicated before spread plating on trypticase soy agar supplemented with 5% whole bovine blood. After an overnight incubation at 37°C, colony-forming units of each culture plate were enumerated to calculate a percentage reduction in colony-forming units, and therefore, the bactericidal activity of PMN. Control cultures did not contain PMN or BsAb. Each test culture was assayed in triplicate.

Statistical Analysis

Comparisons of respiratory burst activity of PMN among treatment groups were analyzed using the

mixed model procedures for measures repeated over time (2). Chemiluminescence activity was expressed as a respiratory burst index and calculated by subtracting counts per minute of mAb-treated cells from counts per minute of untreated cells. Bactericidal activity of PMN was expressed as a percentage reduction in colony-forming units and calculated by the formula:

$$\text{Percent reduction} = A - B / A$$

where:

A = number of colony-forming units recovered from control cultures that did not contain PMN or BsAb;
B = number of colony-forming units recovered from test cultures which contained PMN or BsAb.

Data were analyzed using the general linear models procedure of SAS and tested by least squares ANOVA (7).

RESULTS

Bispecific antibodies were created by chemically coupling two purified mAb. Bispecific antibodies were formed by labeling lysine residues of PMN and Cp5 mAb with N-succinimidyl-3-(2-pyridithiol)-propionate to form reducible disulfide bonds (4, 11). The disulfide bonds of one labeled mAb were reduced and then reacted with the other unreduced labeled mAb to form bispecific heteroaggregates of antibodies. Crosslinked heteroaggregates of antibodies were purified by size-exclusion liquid chromatography, where BsAb (fraction I) eluted before 150 kDa and monomeric mAb (fraction II) eluted at 150 kDa. Elution profiles of BsAb 11G10 × Cp5, 36H10 × Cp5, and 6C6 × Cp5 are shown on Figures 1, 2, and 3, respectively. The efficiency of chemically coupling mAb monomers with N-succinimidyl-3-(2-pyridithiol)-propionate was approximately 50% for each BsAb produced. The amounts of 11G10 × Cp5, 36H10 × Cp5, and 6C6 × Cp5 produced were 580, 670, and 870 μg, respectively.

The modulations of PMN respiratory burst activity by PMN mAb 36H10 and 6C6 are shown in Figures 4 and 5, respectively. The mAb 36H10 enhanced ($P < 0.05$) native chemiluminescence activity of PMN at 15 min, which persisted for the duration of the assay. The mAb 6C6 (Figure 5) enhanced ($P < 0.05$) respiratory burst activity at 30 min. This trend continued over the 120 min. The *Strep. agalactiae* mAb also induced native chemiluminescence activity of PMN by possible binding of Ig and complement in the ascites to Fc and complement receptors on PMN. However, the respiratory burst index of *Strep. agalactiae* mAb stimulated PMN was lower ($P < 0.05$) than the respiratory burst index of either 36H10 or 6C6 treated PMN. The stimulation index of mAb 36H10 and 6C6 was 2.5- and 2.3-fold higher, respectively, than the stimulation index of PMN activated by *Strep. agalactiae* mAb.

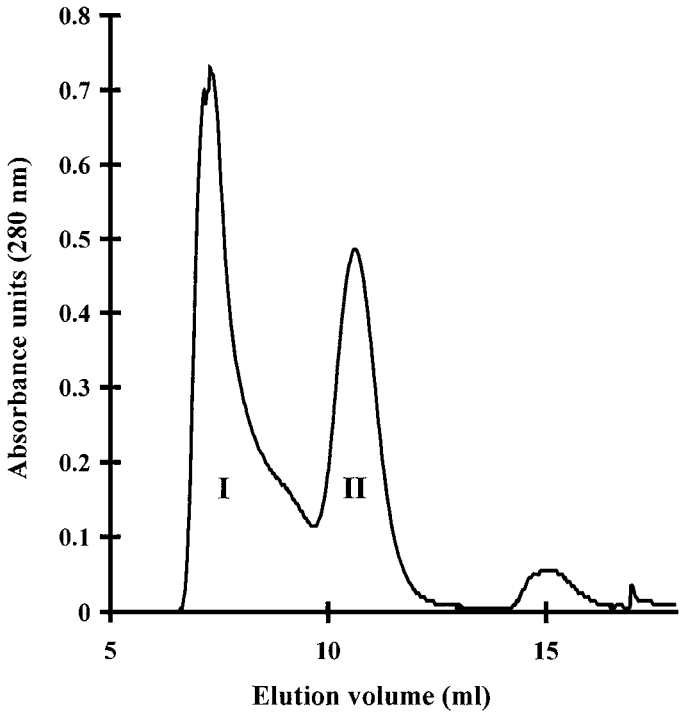

Figure 1. Size-exclusion elution profile of the bispecific antibody (BsAb) 11G10 × Cp5. The BsAb was prepared by coupling bovine polymorphonuclear neutrophilic leukocyte (PMN) monoclonal antibody (mAb) 11G10 and *Staphylococcus aureus* mAb Cp5 with succinimidyl-3-(2-pyridyldithiol)-propionate, and purified by gel filtration. Fraction I contains the BsAb 11G10 × Cp5, and fraction II contains monomers of PMN and Cp5 mAb.

An assay was conducted to determine the effect of *S. aureus* 305 to PMN ratio on the bactericidal activity of PMN at constant concentration of BsAb (Table 1). In the absence of BsAb in bacteria-PMN cultures, a reduction ($P < 0.05$) of 8.7 and 10.1% in colony-forming units was observed at *S. aureus* to PMN ratios of 1:100 and 1:500, respectively. Results from a preliminary study showed that the addition of BsAb or monomers of PMN or Cp5 mAb to *S. aureus* 305 cultures without PMN did not affect the number of *S. aureus* 305 cfu (data not shown). Differences ($P < 0.05$) among BsAb types within a *S. aureus* 305 to PMN ratio were observed at a ratio of 1:500. The BsAb 36H10 × Cp5 induced the highest reduction in colony-forming units (33.4%) followed by 11G10 × Cp5 (26.8%) and C6C × Cp5 (15.1%). An opti-

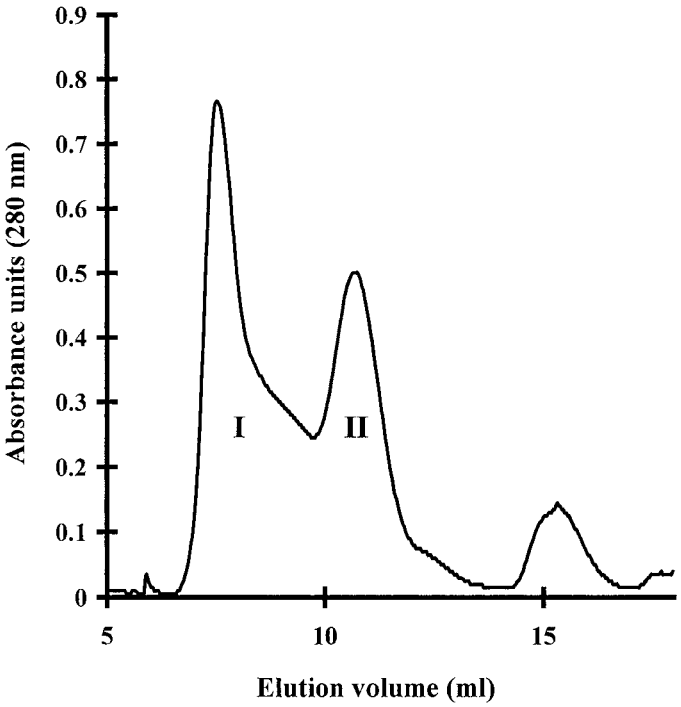

Figure 2. Size-exclusion elution profile of the bispecific antibody (BsAb) 36H10 × Cp5. The BsAb was prepared by coupling bovine polymorphonuclear neutrophilic leukocyte (PMN) monoclonal antibody (mAb) 36H10 and *Staphylococcus aureus* mAb Cp5 with succinimidyl-3-(2-pyridyldithiol)-propionate, and purified by gel filtration. Fraction I contains the BsAb 36H10 × Cp5, and fraction II contains monomers of PMN and Cp5 mAb.

mal *S. aureus* 305 to PMN ratio of 1:500 was observed for each BsAb tested.

The effect of increasing the concentration of BsAb on the bactericidal activity of PMN while maintaining a constant *S. aureus* 305 to PMN ratio of 1:500 is shown in Table 2. Increasing the concentration of each BsAb type from 10 to 50 μg did not potentiate the bactericidal activity of PMN ($P > 0.05$). A consistent level of reduction in colony-forming units was observed within each BsAb type. However, preincubation of *S. aureus* 305 with 10 μg or greater of 36H10 × Cp5 or 6C6 × Cp5 induced maximal bacterial kill by PMN ($P < 0.05$) compared with a concentration of 5 μg. At a concentration of 50 μg, BsAb 36H10 × Cp5 induced the highest percentage reduction ($P < 0.05$) in colony-forming units by PMN (26.9%) followed by 6C6 × Cp5 (23.4%) and 11G10 × Cp5 (20.5%). The ability to increase the bactericidal activity of PMN with different types of BsAb was not different ($P > 0.05$) at concentrations of 5, 10, or 20 μg.

DISCUSSION

Bovine mastitis continues to be the major contributor to economic losses in the dairy industry despite advances in herd health management practices. The standard practice for the treatment of mastitis has been the intramammary administration of antibiotics into infected quarters. While this method of treatment has been shown to be effective against several pathogens that infect the mammary gland, continued use of antibiotics can create new problems for the dairy industry such as contamination of milk products and the selection of antibiotic resistant strains of bacteria (12, 15, 16). Therefore, novel methods of treatment must be developed as alternatives to antibiotic therapy against intramammary infection.

The PMN is the primary leukocyte involved in the defense of the mammary gland and is capable of eliminating bacteria by phagocytosis or antibody-dependent cellular cytotoxicity (5, 9, 13). The bactericidal activities of PMN are exerted through a respiratory burst reaction that produces reactive oxygen species of hydroxyl and oxygen radicals (1, 13). However, this mode of killing is random and inefficient due to the lack of recognition between PMN and bacteria.

Bispecific antibodies have been shown to be effective agents in immunotherapy when the redirection of cellu-

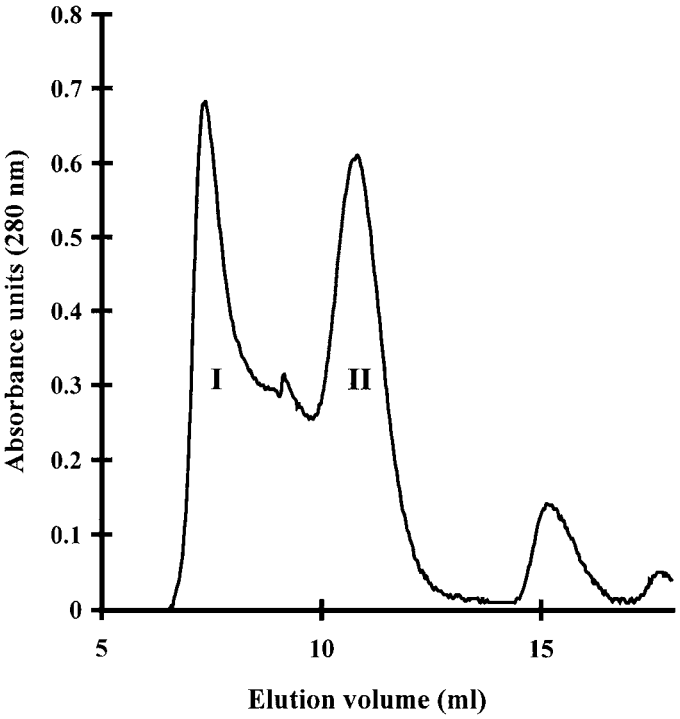

Figure 3. Size-exclusion elution profile of the bispecific antibody (BsAb) 6C6 × Cp5. The BsAb was prepared by coupling bovine polymorphonuclear neutrophilic leukocyte (PMN) monoclonal antibody (mAb) 6C6 and *Staphylococcus aureus* mAb Cp5 with succinimidyl-3-(2-pyridyldithiol)-propionate, and purified by gel filtration. Fraction I contains the BsAb 6C6 × Cp5, and fraction II contains monomers of PMN and Cp5 mAb.

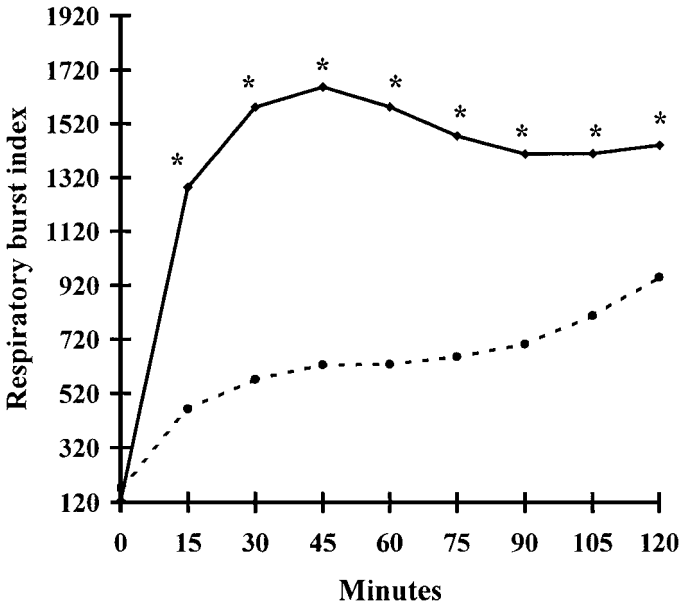

Figure 4. Respiratory burst index of polymorphonuclear neutrophilic leukocytes (PMN) stimulated by 36H10 (♦) or *Streptococcus agalactiae* (●) monoclonal antibodies (mAb). Respiratory burst index is expressed as counts per minute of luminol amplified native chemiluminescence of stimulated cells minus the counts per minutes of nonstimulated cells. Counts per minute were recorded at 15-min intervals for 120 min. Asterisks indicate a significant difference ($P < 0.05$) between 36H10 mAb and *Strep. agalactiae* mAb within minutes. Blood PMN were collected from 2 cows. Each assay was performed in duplicate.

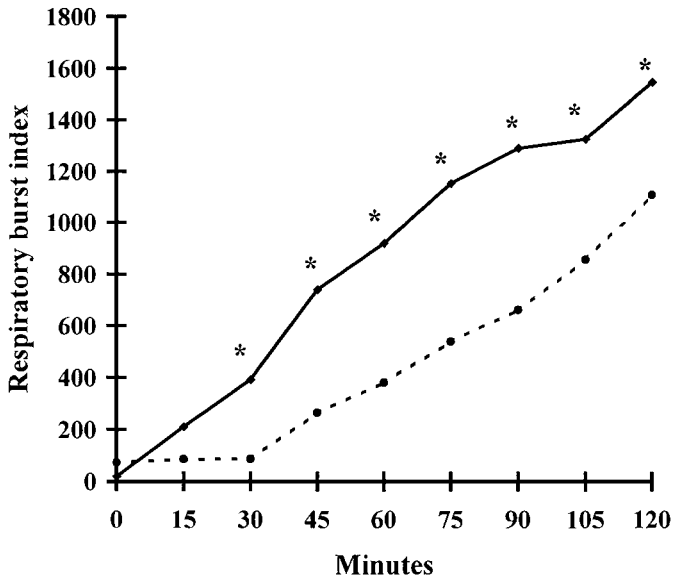

Figure 5. Respiratory burst index of polymorphonuclear neutrophilic leukocytes (PMN) stimulated by 6C6 (♦) or *Streptococcus agalactiae* (●) monoclonal antibodies (mAb). Respiratory burst index is expressed as counts per minute of luminol amplified native chemiluminescence of stimulated cells minus the counts per minutes of nonstimulated cells. Counts per minute were recorded at 15-min intervals for 120 min. Asterisks indicate a significant difference ($P < 0.05$) between 6C6 mAb and *Strep. agalactiae* mAb within minutes. Blood PMN were collected from 2 cows. Each assay was performed in duplicate.

lar cytotoxic defense mechanisms is needed (4, 11). The use of BsAb in cancer therapy has enhanced immunity by specifically targeting tumor cells to effector cells, target toxins, or tumor cell enzymes. The current study investigated the use of BsAb as an immunotherapeutic agent against a pathogen that infects the mammary gland. Bispecific antibodies were used to essentially crosslink bacteria to PMN. This allowed the PMN to be in close physical proximity to bacteria and specifically stimulated the respiratory burst activity of PMN, a major bactericidal mechanism.

A recent study (14) has shown that the mAb 11G10 recognizes the adhesion receptor, L-selectin, on the cell membrane of bovine PMN. An up-regulation effect of 11G10 binding to PMN on respiratory burst activity was detected by an increase in chemiluminescence. The increase in luminol-amplified native chemiluminescence was attributed to the generation of superoxide anions and the formation of oxygen derivatives following the binding of mAb to PMN (1, 8). In the current study, similar activities were observed when PMN were incubated with mAb 36H10 and 6C6. A peak in the respiratory burst index was induced by mAb 36H10 after 45 min of incubation and remained at a constant elevated level for the duration of the assay, while a continuous increase in native respiratory burst activity was observed in PMN treated with mAb 6C6. Although an up regulation of respiratory burst activity was noted, the specific receptor by which mAb 36H10 and 6C6

Table 1. Effect of *Staphylococcus aureus* to polymorphonuclear neutrophilic leukocyte (PMN) ratio on the bactericidal activity of PMN. Bacteria were preincubated with 5 μg of bispecific antibodies (BsAb) and various concentrations of PMN were added to culture wells. Data represents least squares means of percentage reduction in colony-forming units. Each assay was performed in triplicate.

	Mean % reduction in cfu[1]			
Ratio[2]	No BsAb	11G10 × Cp5[3]	36H10 × Cp5	6C6 × Cp5
1:10	0.2^{a}	5.0^{a}	3.8^{a}	2.5^{a}
1:100	8.7^{b}	10.8^{ab}	11.6^{b}	8.6^{ab}
1:500	10.1^{b}‡	26.8^{b}*†	33.4^{b}*	15.1^{b}†

[a,b]Column means with different superscript differ ($P < 0.05$).

[1]Percentage reduction = [(number of cfu recovered from control cultures that did not receive PMN – number of cfu recovered from cultures that received PMN) / number of cfu recovered from control cultures that did not receive PMN] × 100.

[2]*Staphylococcus aureus* to PMN ratio.

[3]BsAb type. 11G10, 36H10, or 6C6 = PMN mAb; Cp5 = *S. aureus* mAb. x = coupling of mAb to form BsAb.

*,†,‡Row means with different superscript differ ($P < 0.05$).

Table 2. Effect of increasing concentration of bispecific antibodies (BsAb) on the bactericidal activity of polymorphonuclear neutrophilic leukocytes (PMN). *Staphylococcus aureus* was preincubated with 0, 5, 10, 20, or 50 μg of BsAb before the addition of PMN to culture wells. The *S. aureus* to PMN ratio was maintained at 1:500. Data represent least squares means of percentage reduction in colony-forming units. Each assay was performed in triplicate.

	Mean % reduction cfu[1]		
μg BsAb[2]	11G10 × Cp5[3]	36H10 × Cp5	6C6 × Cp5
5	18.1^{ab}	16.3^{a}	18.3^{a}
10	21.9^{abc}	25.2^{bcd}	23.9^{bcd}
20	25.0^{bcd}	21.8^{bc}	23.6^{bcd}
50	20.5^{abd}†	26.9^{bd}*	23.4^{bcd}*†

^{a,b,c,d}Column means with different superscript differ ($P < 0.05$).

[1]Percentage reduction = [(number of cfu recovered from control cultures that did not receive BsAb – number of cfu recovered from cultures that received BsAb) / number of cfu recovered from control cultures that did not receive BsAb] × 100.

[2]Concentration of BsAb added to wells.

[3]BsAb type. 11G10, 36H10, or 6C6 = PMN mAb; Cp5 = *S. aureus* mAb. x = indicate coupling of mAb to form BsAb.

*,†Row means with different superscript differ ($P < 0.05$).

modulates PMN respiratory burst activity is not known. An increase in oxygen metabolism was also detected when PMN were incubated with the *Strep. agalactiae* control mAb, although the respiratory burst index was lower than mAb 36H10 or 6C6 stimulated PMN. This observation was attributed to nonspecific binding of *Strep. agalactiae* control mAb, Ig, and complement in ascites to Fc and complement receptors on PMN (8, 14).

In the present study, *S. aureus* 305 was used as a mastitis pathogen model to determine the influence of BsAb on in vitro bactericidal activities of PMN. Preincubation of *S. aureus* 305 with 5 μg of BsAb increased the bactericidal activity of PMN by 1.5- to 3-fold at a *S. aureus* 305 to PMN ratio of 1:500. Each BsAb type exhibited the ability to enhance the reduction of colony-forming units at a ratio of 1:500 compared with the absence of BsAb. Differences were not observed within a BsAb type as a function of increasing the *S. aureus* 305 to PMN ratio from 1:100 to 1:500. At a ratio of 1:500, the percent reduction in colony-forming units among BsAb types was higher than that observed in the absence of BsAb. This observation may be attributed to the specific and efficient crosslinking of bacteria to PMN by BsAb and subsequent modulation of the respiratory burst activity, and not the nonspecific killing of bacteria by random contact with PMN. An increase in bactericidal activity due to the presence of BsAb was not observed at *S. aureus* 305 to PMN ratios of 1:10 or 1:100. However, a reduction in colony-forming units was noted in the absence of BsAb at those ratios and was attributed to nonspecific killing by PMN.

Increasing the concentration of BsAb while maintaining a constant *S. aureus* 305 to PMN ratio was also shown to decrease the number of *S. aureus* 305 in cultures. A maximal reduction in colony-forming units was noted when *S. aureus* 305 was preincubated with greater than 5 μg of BsAb. Preincubation with 10, 20, or 50 μg of all BsAb types did not appreciably enhance the bactericidal activity of PMN. Therefore, a *S. aureus* 305 to PMN ratio of 1:500 and a concentration of 10 μg of BsAb may represent optimal bactericidal efficiency by PMN within the context of the assay in this study. Differences among BsAb types were observed at a concentration of 50 μg. The BsAb 36H10 × Cp5 elicited the highest reduction in colony-forming units, followed by BsAb 6C6 × Cp5 and 11G10 × Cp5. These results paralleled the finding of the ability of PMN mAb to modulate the native chemiluminescence of PMN. At 60 min of incubation, which was the incubation time of the bactericidal assay, the PMN mAb 36H10 induced a 2.5-fold greater respiratory burst index than the respiratory burst index of PMN stimulated by the *Strep. agalactiae* control mAb. This was followed by C6C and 11G10 [data from Wang et al. (14)], which exhibited a 2.3- and 2.1-fold increase in the respiratory burst index, respectively. Therefore, the results indicate that the mAb that induced the greatest respiratory burst activity also enhanced the highest degree of bactericidal activity in PMN.

In conclusion, results from the current study suggest that BsAb immunotherapy may serve as an alternative to antibiotic treatment against bovine mastitis and may also be used to target and kill antibiotic-resistant pathogens that infect the mammary gland. Monoclonal antibodies against bovine PMN were shown to enhance the respiratory burst activity of PMN, and the mAb that elicited the greatest respiratory burst response also induced the greatest bactericidal activity of PMN when coupled to mAb Cp5. A two- to threefold increase in PMN bactericidal activity was observed when *S. aureus* 305 was preincubated with BsAb. Bispecific antibodies also enhanced the bactericidal activity of PMN in a dose dependent manner. The concept of enhancing the immune function of bovine PMN with BsAb has been demonstrated in this study and represents a preliminary investigation and a novel approach to treat bovine mastitis.

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